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The Xenopus Cdc6 Protein Is Essential for the Initiation of a Single Round of DNA Replication in Cell-Free Extracts

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Summary

We have cloned a Xenopus Cdc6 homolog (Xcdc6) and characterized its role in DNA replication with Xenopus egg extracts. Immunodepletion of Xcdc6 abolishes chromosomal replication but not elongation on single-stranded DNA templates. Xcdc6 binds to chromatin at the beginning of interphase but disappears from chromatin upon initiation of replication. Immunodepletion studies indicate that binding of Xcdc6 to chromatin requires Xorc2, a component of the origin recognition complex. Moreover, Xmcm3 cannot bind to chromatin lacking Xcdc6, suggesting that Xorc2, Xcdc6, and Xmcm3 associate with the DNA sequentially. In postreplicative nuclei, Xcdc6 is associated with the nuclear envelope. These studies indicate that Xcdc6 is essential for initiation of replication in vertebrates and that interaction with the nuclear envelope may regulate its function.

Introduction

In all eukaryotic cells, the replication and segregation of the chromosomes must be controlled rigorously during each cell division cycle. In the case of DNA replication, several regulatory processes ensure that chromosomal duplication occurs in a faithful manner. In a typical somatic cell, one or more cyclin-dependent kinases (Cdks) trigger the firing of replication origins at the beginning of S-phase once the cell has grown to a critical minimum size and accumulated an adequate supply of the molecular components necessary for DNA synthesis (reviewed in Coverley and Laskey, 1994; Heichman and Roberts, 1994). Another essential feature of S-phase is that each segment of chromosomal DNA must be replicated only once in each cell cycle. This regulatory process, often called the "block-to-rereplication," most likely involves a unidirectional switch or licensing factor at replication origins that can be utilized productively only once during each replicative cycle (Coverley and Laskey, 1994; Heichman and Roberts, 1994; Chong et al., 1996). Finally, it is also critical that DNA replication be completed punctually so that partially replicated chromosomes are not incorporated into the mitotic spindle prior to cell division. Currently, it is thought that during S-phase one or more active replication proteins communicate with the cell cycle control machinery to enforce a "replication checkpoint" that guards against aberrant cell division (Hartwell and Weinert, 1989; Kornbluth et al., 1992; Kelly et al., 1993).

The regulation of DNA replication and its integration into the cell cycle involve a variety of *cis*-acting proteins that bind to chromosomes prior to and/or during S-phase. Recent genetic and biochemical studies have shown that the origin recognition complex (ORC), an assembly of six proteins that binds specifically to replication origins in budding yeast, is essential for the initiation of replication (Bell, 1995; Bell et al., 1995; Diffley, 1995). Similarly, the budding yeast Cdc6 protein and its fission yeast homolog Cdc18 appear to act at a critical early step in chromosomal replication (Hartwell, 1976; Bueno and Russell, 1992; Kelly et al., 1993; Nishitani and Nurse, 1995; Piatti et al., 1995; Muzi-Falconi et al., 1996a), perhaps by interacting directly with ORC (Liang et al., 1995). Finally, proteins in the minichromosome maintenance (Mcm) family are essential replication factors that associate with chromatin in a dynamic manner throughout the replication cycle in both yeast and vertebrates (reviewed in Chong et al., 1996).

In budding yeast, ORC is bound to replication origins throughout the cell cycle, but the association of other proteins with the origin appears to vary in a dynamic manner. In particular, the footprint of proteins on the DNA replication origin varies from a pre-replicative to a postreplicative form during the course of the cell cycle (Brown et al., 1991; Diffley et al., 1994). The formation of the pre-replicative state, which occurs at the end of mitosis, requires the presence of the Cdc6 protein, suggesting that Cdc6 is a component of the preinitiation complex (Cocker et al., 1996). During S-phase, the prereplicative complex switches to a postreplicative form, which presumably would lack the Cdc6 protein. A variety of studies have indicated that active Cdks containing B-type cyclins prevent the reestablishment of the prereplicative complex until the end of the subsequent mitosis (Adachi and Laemmli, 1994; Hayles et al., 1994; Dahmann et al., 1995; Jallepalli and Kelly, 1996). These and related observations have led to the concept that the formation and activation of the prereplicative complexes govern the proper utilization of replication origins in S-phase (reviewed in Diffley, 1995; Wang and Li, 1995; Muzi-Falconi et al., 1996b).

Extracts from Xenopus eggs have proven to be invaluable for studying the biochemical mechanisms of eukaryotic chromosomal replication (Blow and Laskey, 1986; Newport, 1987). In this system, frog chromosomes undergo a complete round of semiconservative DNA synthesis in approximately one hour. Moreover, this replication process occurs in a properly regulated manner: the chromosomes undergo only one round of replication during each in vitro cell cycle, and the duplicating chromosomes can elicit the mobilization of the replication checkpoint (Blow and Laskey, 1986; Dasso and Newport, 1990). In recent years, a number of essential replication factors that appear to act at early regulatory steps have been identified and characterized in the Xenopus system. These include replication protein A (RPA), Xmcm3 and other members of the Mcm family, and Xorc2, a component of the origin recognition complex (Adachi and Laemmli, 1994; Carpenter et al., 1996; Chong et al., 1996). In this report, we have isolated a

frog homolog of the Cdc6 protein (Xcdc6) and examined how it collaborates with Xorc2, Xmcm3, and RPA in controlling the faithful replication of Xenopus chromosomes in egg extracts. Our findings demonstrate that Xcdc6 fulfills a critical early function in DNA replication. The properties of Xcdc6 indicate that it may play a role in the regulatory system that limits replication to one round per cell cycle.

Results

Isolation of a Xenopus Cdc6 Homolog

To clone a potential Xenopus Cdc6 homolog, we used degenerate polymerase chain reaction (PCR) primers to amplify a segment of its complementary DNA (cDNA). The primers were designed on the basis of sequence homology within conserved regions of the S. cerevisiae Cdc6 and S. pombe Cdc18 proteins. PCR amplification yielded an ~800 bp fragment that was used to isolate a full-length Xenopus oocyte cDNA, which encodes a protein with strong homology to the Cdc6/Cdc18 family (GenBank accession no. U66558). The open reading frame, which is preceded by three in-frame termination codons, encodes a 554 amino acid polypeptide (Xcdc6) with a predicted M_r of 61,000 Daltons.

Xcdc6 shares significant sequence identity with both the S. cerevisiae Cdc6 (30% identical residues) (Liszewicz et al., 1988; Zhou et al., 1989) and S. pombe Cdc18 proteins (34%) (Kelly et al., 1993). Xcdc6, like S. cerevisiae Cdc6 and S. pombe Cdc18, contains a consensus purine nucleotide binding motif (GXXGXGKT, residues 196–203). Moreover, Xcdc6 contains ten Ser-Pro or Thr-Pro motifs that reside in potential recognition sites for Cdks and other cell cycle regulated kinases. Finally, Xcdc6 contains a putative bipartite nuclear localization signal (KKTSQTLAKEVSRAKSK, residues 17–33).

Characterization of the Xcdc6 Protein in Xenopus Egg Extracts

To characterize the role of Xcdc6 in DNA replication and cell cycle control in Xenopus egg extracts, we expressed a six histidine-tagged version of Xcdc6 (His6-Xcdc6) in baculovirus-infected Sf9 insect cells. The recombinant His6-Xcdc6 protein was isolated by nickel-agarose chromatography, and the purified protein (Figure 1A, lane 4) was injected into rabbits for polyclonal antibody production. In immunoblotting experiments, these antibodies recognize a single 61 kDa polypeptide in interphase extracts from Xenopus eggs (Figure 1A). Xcdc6 is present at equivalent amounts in M-phase extracts, but in a modified form, as indicated by a reduced electrophoretic mobility (Figure 1A). In other experiments, we established that the level of Xcdc6 is essentially constant throughout the early embryonic cell cycles (data not shown). The concentration of Xcdc6 protein in Xenopus eggs is ~5 ng/μl or 80 nM. This value is comparable to that of both Xenopus RPA (125 nM; Adachi and Laemmli, 1994) and Xorc2 (100 nM; Carpenter et al., 1996). Hence, Xcdc6, as is the case with other replication factors, is stored in large amounts for utilization during the rapid cleavage stage of early embryonic development.

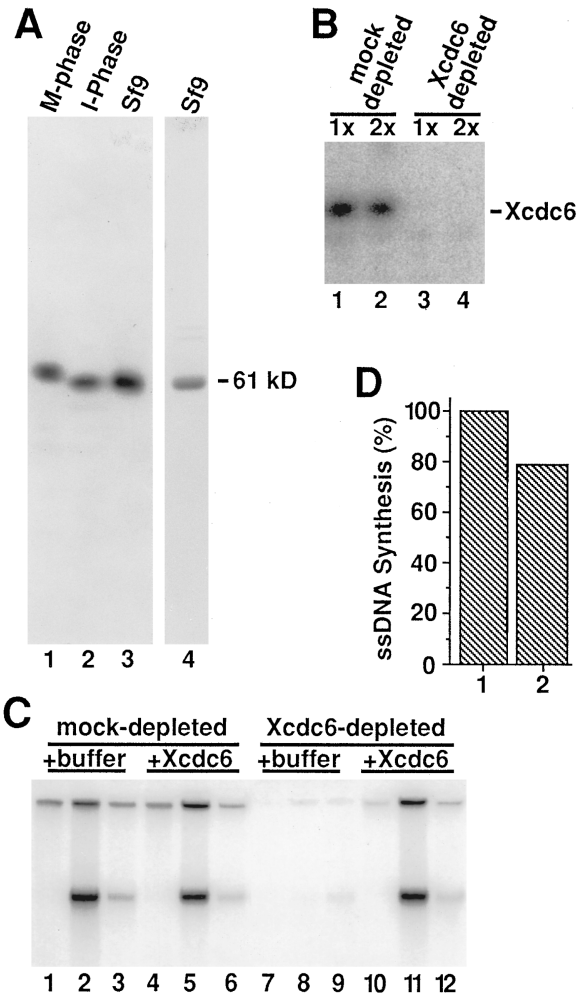

Figure 1. Immunodepletion of Xcdc6 Abolishes Chromosomal Replication in Xenopus Egg Extracts

(A) Immunoblotting with anti-Xcdc6 antibodies (lanes 1–3) of Xenopus CSF-arrested extract (M phase: lane 1, 25 μg), interphase extract (I phase: lane 2, 25 μg), and purified histidine-tagged Xcdc6 protein (Sf9: lane 3, 5 ng protein). In lane 4, purified His6-Xcdc6 protein (3 μg) has been stained with Coomassie blue.

(B) Xcdc6 can be immunodepleted quantitatively from interphase egg extracts. Immunoblotting with anti-Xcdc6 antibodies of interphase extracts that had been treated once (1x) or twice (2x) with either control (lanes 1 and 2) or anti-Xcdc6 (lanes 3 and 4) antibodies bound to protein A–agarose.

(C) Replication of sperm chromatin was determined in mock-depleted (lanes 1–6) or Xcdc6-depleted (lanes 7–12) interphase extracts in the presence of either control buffer or purified His6-Xcdc6 (5 ng/μl). Demembranated sperm chromatin was added along with either control buffer (lanes 1–3 and 7–9) or purified His6-Xcdc6 (lanes 4–6 and 10–12) immediately following the immunodepletion procedure. The samples were warmed to 23°C, and aliquots were pulse labeled with [α-^{32}P]dCTP at successive 30 min intervals (see Experimental Procedures). Each lane depicts the ^{32}P incorporation into chromosomal DNA which occurred at the following times: 1–30 min (lanes 1, 4, 7, and 10); 30–60 min (lanes 2, 5, 8, and 11); and 60–90 min (lanes 3, 6, 9, and 12).

(D) Quantitation of single-stranded M13 DNA replication during a 90 min incubation at 23°C in either a mock-depleted (column 1) or Xcdc6-depleted (column 2) extract. The efficiency of Xcdc6 depletion was verified by immunoblotting (part C, 91%; part D, 96%).

Xcdc6 Is Essential for Chromosomal Replication in Xenopus Egg Extracts

Both budding yeast Cdc6 and fission yeast Cdc18 have been implicated as essential factors for the initiation of DNA replication, but much remains to be learned about their biochemical function (Hartwell, 1976; Kelly et al., 1993; Nishitani and Nurse, 1995). The replication of sperm chromatin in Xenopus egg extracts represents a valuable system for elucidating the mechanisms of eukaryotic chromosomal replication (Blow and Laskey, 1986; Newport, 1987). To investigate the role of Xcdc6 in DNA replication with this system, we removed Xcdc6 by immunodepletion with anti-Xcdc6 antibodies. For this purpose, we treated interphase egg extracts with protein A beads bound to anti-Xcdc6 antibodies or rabbit anti-mouse IgG control antibodies. This procedure resulted in the removal of 90%–98% of Xcdc6 from extracts treated with the anti-Xcdc6 antibody (see Figure 1B). Next, we added sperm chromatin and [α-^{32}P]dCTP to the Xcdc6-depleted and control-depleted extracts and determined the rate and extent of chromosomal DNA synthesis. We observed that incorporation of ^{32}P into chromosomal DNA was severely diminished in the Xcdc6-depleted extract relative to the control extract (Figure 1C, compare lanes 7–9 with lanes 1–3). In the experiment shown in Figure 1C, DNA replication in the extract lacking Xcdc6 was reduced to only 4% of the control value. This inhibition of DNA replication cannot be attributed to a defect in nuclear assembly, which is essential for chromosomal replication in Xenopus egg extracts (Blow and Laskey, 1986; Newport, 1987), since nuclear envelope formation proceeded with indistinguishable kinetics in both the Xcdc6-depleted and control extracts (data not shown). Finally, to characterize the replication defect in the Xcdc6-depleted extracts further, we examined whether these extracts could carry out replication of single-stranded M13 DNA, a process that does not require replication origins (Méchali and Harland, 1982; Carpenter et al., 1996). The Xcdc6-depleted extracts were found to replicate M13 DNA efficiently (80% relative to control extracts; Figure 1D), indicating that Xcdc6 is not required for elongation on a single-stranded DNA template.

An important issue is whether the replication defect in the Xcdc6-depleted extract can be restored by the addition of recombinant Cdc6 protein (Figure 1C). Indeed, when we added back recombinant His6-Xcdc6 at the physiological concentration of 80 nM, we observed that DNA replication in an extract that had been treated with anti-Xcdc6 antibodies could be restored to $\sim$85% of the control level (Figure 1C, lanes 10–12). This finding indicates that removal of Xcdc6 from the extracts does not inactivate the replicative capacity of the extracts irreversibly. Moreover, this observation indicates that Xcdc6, in contrast to Xorc2 (Carpenter et al., 1996), is not associated quantitatively with another essential replication protein(s) in egg extracts. Another notable feature of these experiments is that the addition of recombinant His6-Xcdc6 protein to a control extract did not stimulate replication above normal levels (96% of control; Figure 1C, lanes 4–6), indicating that overexpression of Xcdc6 in this system does not disrupt the regulatory system which ensures that DNA replication occurs only once per cell cycle. This situation contrasts with that in fission yeast, where overexpression of Cdc18 triggers multiple aberrant rounds of DNA replication (Nishitani and Nurse, 1995; Muzi-Falconi et al., 1996a). The difference may be due to the fact that Xcdc6 has been stored in large amounts in the egg, implying that the block to rereplication during early Xenopus embryogenesis can operate properly in the presence of high concentrations of Xcdc6.

Xcdc6 Is Eliminated from Chromatin upon the Initiation of DNA Replication

Collectively, the above experiments indicate that the removal of Xcdc6 from Xenopus egg extracts specifically abolishes an early step in chromosomal replication. To examine the functional properties of Xcdc6 in greater detail, we examined its localization at various stages of DNA replication in the Xenopus egg extracts. For this purpose, we utilized both indirect immunofluorescence and immunoblotting with anti-Xcdc6 antibodies to examine the association of Xcdc6 with chromatin and other nuclear structures during the replication cycle (Figure 2). In parallel, we also examined the binding of two other critical replication factors, Xorc2 and Xmcm3, to nuclei with antibodies against these proteins.

In the first set of experiments, we examined the binding of Xcdc6 to sperm chromatin as a function of time in Xenopus egg extracts. As determined by indirect immunofluorescence (data not shown) and immunoblotting with anti-Xcdc6 antibodies (Figure 2C, top panel, lanes 2 and 3), the binding of Xcdc6 to chromatin was severely diminished in CSF-arrested egg extracts, which are blocked in M phase. Similarly, we observed that there was no binding of the Xorc2 protein to chromatin in CSF extracts, as indicated by indirect immunofluorescence (data not shown) and immunoblotting with anti-Xorc2 antibodies (Figure 2C, middle panel, lanes 2 and 3). Furthermore, neither Xorc2 nor Xcdc6 could be found on chromatin from nuclei that had undergone disassembly at the first mitosis after activation of the egg extracts (Figure 2B, bottom panels; Figure 2C, top and middle panels, lane 9). Thus, in the Xenopus system, neither Xorc2 nor Xcdc6 can associate with chromatin at M phase. The situation is different in S. cerevisiae, where the origin recognition complex (presumably containing the Orc2 protein) binds to the DNA throughout the cell cycle (Bell, 1995; Diffley, 1995).

After adding Ca^{2+} to the egg extracts in order to drive them into interphase, we observed that over a 30 min period Xcdc6 gradually associated with chromatin, as indicated by both indirect immunofluorescence and immunoblotting with anti-Xcdc6 antibodies (Figure 2A; Figure 2C, top panel). During this time, nuclear envelope assembly occurs around the chromatin and DNA replication begins to initiate within the assembled nuclei. Throughout this period, immunofluorescence staining of Xcdc6 on the chromatin was relatively uniform, showing no indication of the punctate staining pattern that has been observed with antibodies against the single-stranded DNA binding replication protein A (RPA) (Adachi and Laemmli, 1992). Similarly, immunofluorescence staining with anti-Xorc2 antibodies also revealed a uniform staining pattern at this time (Carpenter et al., 1996).

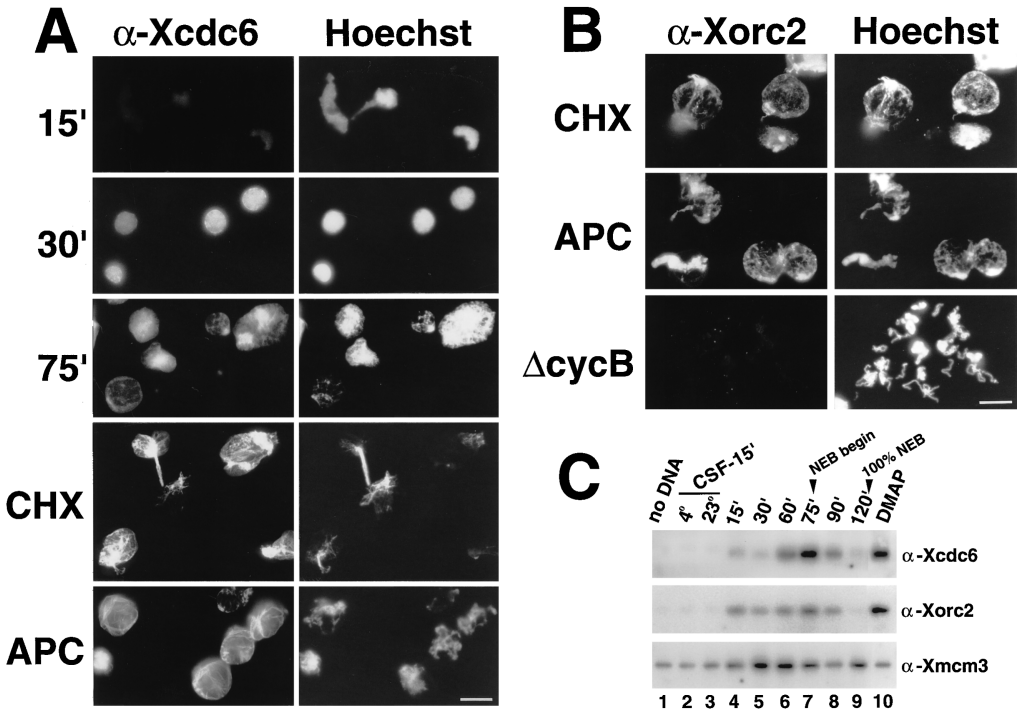

Figure 2. Xcdc6 Associates with Chromatin in a Dynamic Manner throughout the Cell Cycle in Xenopus Egg Extracts

(A) Demembranated sperm chromatin was incubated in standard interphase extracts for the indicated times (top six panels) or interphase extracts containing cycloheximide (CHX) or aphidicolin (APC) for 120 min (bottom four panels). Samples were visualized by indirect immunofluorescence with anti-Xcdc6 antibodies (left panels) or by staining with Hoechst 33258 (right panels). Magnification bar, 10 μm.

(B) Xorc2 localizes to the chromatin during interphase but abruptly leaves upon entry into mitosis. Demembranated sperm chromatin was incubated in interphase extracts containing either cycloheximide (CHX) or aphidicolin (APC) for 90 min. In a parallel experiment, an aliquot of the cycloheximide-treated extract was driven into mitosis by adding Δcyclin B and incubating an additional 10 min. Samples were visualized by indirect immunofluorescence with anti-Xorc2 antibodies (left panels) or by staining with Hoechst 33258 (right panels). Magnification bar, 10 μm.

(C) Immunoblots were performed on the insoluble pellet fraction from unactivated (CSF, lanes 2 and 3) or interphase (lanes 1 and 4–10) extracts in the absence (lane 1) or presence of demembranated sperm chromatin (lanes 2–10). Samples were incubated at 4°C (lane 2) or 23°C (lanes 1 and 3–10) for the indicated times and then prepared for immunoblot analysis using anti-Xcdc6 (top panel), anti-Xorc2 (middle panel), or anti-Xmcm3 (bottom panel) antibodies. The sample in lane 10 was treated with the kinase inhibitor DMAP for 30 min. Since the entry into interphase occurs more rapidly in the presence of DMAP, the binding of Xorc2 and Xcdc6 is accelerated relative to the control extract. However, the binding of Xmcm3 is essentially at background levels in the control and DMAP-treated extract. It is important to point out that for interphase nuclei these immunoblots detect proteins bound not only to chromatin but also to the detergent-insoluble matrix that surrounds the chromatin. Cell cycle progression of the extracts was monitored visually by assessing nuclear envelope breakdown (NEB).

Finally, the kinase inhibitor 6-dimethylaminopurine (DMAP) did not inhibit the ability of either Xorc2 or Xcdc6 to associate with chromatin (Figure 2C, top and middle panels, lane 10). Previous studies have shown that DMAP blocks DNA replication in Xenopus egg extracts and inhibits the binding of Xmcm3, a proposed component of replication licensing factor, to the DNA (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995a). As expected, we observed that Xmcm3 could not bind above background levels to DMAP-treated nuclei (Figure 2C, bottom panel, lane 10). Thus, in contrast to Xmcm3, both Xcdc6 and Xorc2 bind chromatin in the presence of DMAP.

Intriguingly, at later times after the activation of the egg extracts (≥75 min), we observed that the Xcdc6 immunofluorescence staining in some nuclei no longer coincided with the chromatin. In particular, in these nuclei anti-Xcdc6 antibodies stained a structure resembling the nuclear envelope. This phenomenon was especially evident in nuclei from extracts that had been treated with either cycloheximide or aphidicolin for 90–120 min (Figure 2A; Figure 3). Treatment with cycloheximide arrests the extracts in interphase by preventing the synthesis of cyclin B, which is essential for mitosis. Aphidicolin, a DNA polymerase inhibitor, blocks replication initiation, but may not inhibit the initial firing of replication origins. This nuclear envelope staining was also observed with a batch of anti-Xcdc6 antibodies that had been purified from a band in an immunoblot containing only the recombinant Xcdc6 protein (not shown). Since these antibodies also recognize only a single 61 kDa band in immunoblots, the staining of the nuclear envelope with anti-Xcdc6 antibodies is unlikely to represent cross-reactivity with another nuclear envelope protein. In strong contrast to Xcdc6, the Xorc2 protein remained associated with chromatin in extracts treated with either cycloheximide or aphidicolin, as indicated by indirect immunofluorescence with anti-Xorc2 antibodies (Figure 2B).

To characterize the apparent binding of Xcdc6 to the nuclear envelope in greater detail, we performed double immunofluorescence with anti-Xcdc6 antibodies and

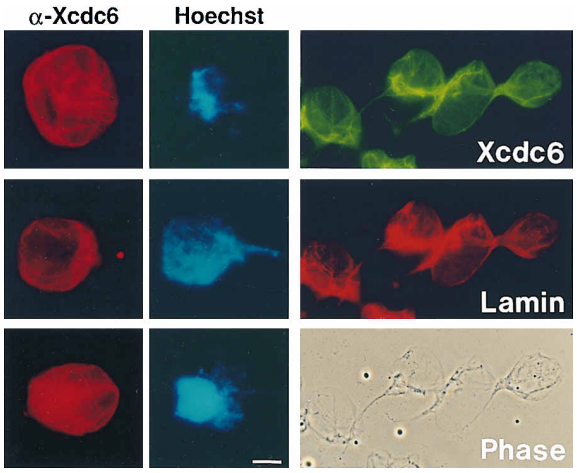

Figure 3. Xcdc6 Is Localized at the Nuclear Envelope in Postreplicative Nuclei

Demembranated sperm chromatin was incubated in an interphase extract containing cycloheximide for 90 min. Three nuclei from one experiment are shown to indicate the range of Xcdc6 (red) and chromatin (blue) staining patterns (left six panels). In a separate experiment, similarly treated nuclei were processed for indirect immunofluorescence using anti-Xcdc6 (green) and anti-lamin L_{III} (red) antibodies. Note that Xcdc6 and lamin colocalize to the nuclear envelope, which is also visible in phase contrast microscopy. Magnification bar, 10 μm.

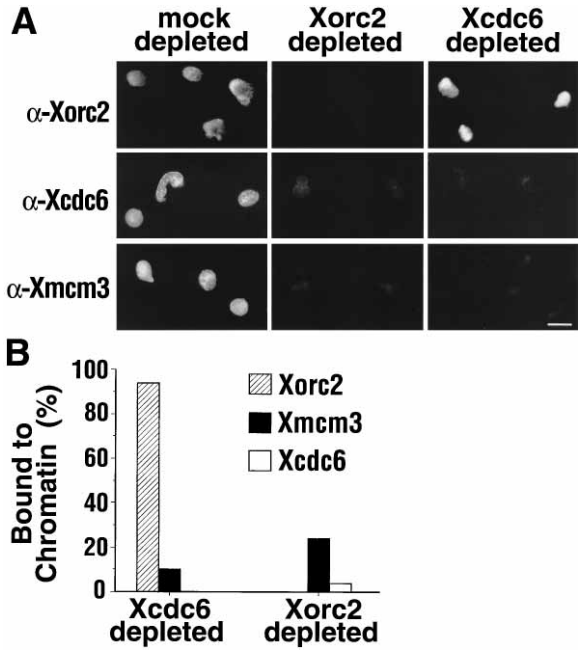

Figure 4. Binding of Xcdc6 to Chromatin Requires Xorc2, and Association of Xmcm3 with Chromatin Is Dependent upon Both Xorc2 and Xcdc6

(A) Demembranated sperm chromatin was incubated for 25 min in each of three interphase extracts: mock-depleted (left column), Xorc2-depleted (middle column), or Xcdc6-depleted (right column). The samples were processed for indirect immunofluorescence with anti-Xorc2 (top row), anti-Xcdc6 (middle row), or anti-Xmcm3 (bottom row) antibodies. Each panel contains from three to five nuclei, as determined by staining with Hoechst 33258 (not shown). Magnification bar, 10 μm.

(B) Chromatin binding of the indicated antigens was determined by quantitating immunoblots of detergent insoluble material isolated from Xcdc6-depleted (left columns), or Xorc2-depleted (right columns) extracts. The values shown were taken from a complete time course experiment and indicate the percentage (relative to the mock-depleted extract) of the various antigens bound to chromatin after warming the extract to 23°C. These percentages were determined when the antigen binding had reached maximal levels in the mock-depleted extracts (typically between 15 and 45 min). All samples were normalized by subtracting the background signals obtained in the detergent-insoluble pellets from extracts that had been incubated in the absence of sperm chromatin. The efficiency of antigen depletion was verified by immunoblotting (part A: Xorc2, 95% depletion; Xcdc6, 95% depletion; part B: Xorc2, 92% depletion; Xcdc6, 98% depletion).

various probes that stain known nuclear envelope constituents. The nuclear envelope is composed of a double-bilayer membrane, the underlying nuclear lamina, and the nuclear pores. We first utilized antibodies against the major Xenopus lamin protein L_{III} (Krohne and Benavente, 1986). We observed that both anti-Xcdc6 and anti-lamin L_{III} antibodies yielded a similar staining pattern in nuclei that had been incubated for 90 min in cycloheximide-treated extracts (Figure 3). In phase contrast microscopy, the structure stained with both the anti-Xcdc6 and anti-lamin antibodies coincided closely with the phase-dense nuclear envelope that surrounded but was distinct from the chromatin (Figure 3). Similar results were obtained when the nuclear envelope was stained with rhodamine-conjugated wheat germ agglutinin (data not shown), a lectin that binds to various glycoproteins in the nuclear pore (Finlay et al., 1987). These observations indicate that Xcdc6 most probably interacts with some component of the nuclear envelope. Electron microscopic studies will be required to explore this issue further.

Collectively, these experiments indicate that Xcdc6 associates with chromatin upon entry into interphase. Thereafter, the Xcdc6 protein is eliminated from the chromatin during the replication process. This elimination occurs even in the presence of aphidicolin, suggesting that Xcdc6 is required for a very early step in DNA replication such as origin unwinding.

Immunodepletion Studies Reveal a Sequential Association of Xorc2, Xcdc6, and Xmcm3 with Chromatin

The above findings indicate that Xorc2, Xcdc6, and Xmcm3 associate with chromatin in a dynamic manner during the cell cycle in Xenopus egg extracts. To examine the potential interdependence in the binding of these proteins to chromatin, we carried out a series of immunodepletion studies. For these experiments, we utilized both indirect immunofluorescence and immunoblotting to examine the ability of these replication proteins to bind to chromatin in either Xorc2-depleted or Xcdc6-depleted extracts (Figure 4). In one set of experiments, we treated interphase extracts with protein A beads bound to either anti-Xorc2 or control antibodies. Subsequently, we incubated sperm chromatin in the various extracts for 25 min, and then performed indirect immunofluorescence on the chromatin with anti-Xorc2, anti-Xcdc6, or anti-Xmcm3 antibodies. We chose an incubation period of 25 min because all three of these proteins

bind to chromatin at this time. As expected, the Xorc2 protein cannot be detected on chromatin that has been incubated in a Xorc2-depleted extract. Significantly, the Xcdc6 and Xmcm3 proteins show dramatically reduced staining on chromatin lacking Xorc2 (Figure 4A, middle column), suggesting that these proteins cannot associate efficiently with chromatin in the absence of Xorc2 and the other presumed components of the Xenopus origin recognition complex. This observation cannot be attributed to the removal of Xcdc6 and Xmcm3 with the anti-Xorc2 antibodies, since immunoblot analysis showed that >90% of both the Xcdc6 and Xmcm3 proteins remained in the extract following immunodepletion of Xorc2 (data not shown).

Next, we performed an analogous set of experiments with Xcdc6-depleted extracts. As would be expected, the staining of chromatin in Xcdc6-depleted extracts with anti-Xcdc6 antibodies is severely diminished. Significantly, the binding of Xorc2 to chromatin in the absence of Xcdc6 is virtually unaffected, whereas the binding of Xmcm3 is strongly diminished (Figure 4A, right column). Moreover, we demonstrated that chromatin that had been incubated in a Xcdc6-depleted extract (to allow binding of Xorc2) could bind Xcdc6 upon transfer to an Xorc2-depleted extract. This observation also argues that Xorc2 and Xcdc6 need not form a complex before binding to chromatin (data not shown).

In a complementary set of experiments, we also used immunoblot analysis of detergent-solubilized nuclei to assess the binding of Xorc2, Xcdc6, and Xmcm3 to chromatin in Xorc2-depleted and Xcdc6-depleted extracts (Figure 4B). For this analysis, we examined the kinetics with which these proteins bound to chromatin in the various extracts, and then directly compared the time of maximal binding in the control extract with the equivalent time point in the depleted extract. The results of these experiments were consistent with those obtained in the immunofluorescence studies. In particular, in the absence of Xcdc6, Xorc2 can bind efficiently to chromatin (96% of control, Figure 4B). In contrast, Xcdc6 cannot associate with chromatin in the absence of Xorc2 (4% of control, Figure 4B). Finally, in the absence of either Xorc2 or Xcdc6, the binding of Xmcm3 to chromatin is markedly reduced (10%–25% of control, Figure 4B). Collectively, these experiments suggest the following interdependencies of these proteins in their ability to associate with sperm chromatin prior to replication: Xcdc6 requires the previous binding of Xorc2, whereas Xmcm3 appears to require both Xorc2 and Xcdc6.

Neither Xorc2 nor Xcdc6 Is Required for the Formation of RPA-Containing Foci

In Xenopus egg extracts and mammalian cells, DNA replication is thought to occur at a discrete number of subnuclear structures or foci (Mills et al., 1989). These foci have been identified either by short pulses with labeled nucleotides or by staining with anti-RPA antibodies. For example, in the Xenopus system, indirect immunofluorescence with anti-RPA antibodies has revealed that RPA associates with several hundred discrete foci within individual nuclei at the beginning of interphase (Adachi and Laemmli, 1992; Jackson et al., 1995; Yan and Newport, 1995). Until recently, the formation of these RPA-containing foci has been viewed as the earliest resolvable step of chromosomal replication in this system. Based upon the results described here and elsewhere (Carpenter et al., 1996), it seems likely that both Xorc2 and Xcdc6 would act upstream of RPA in the process of replication, since RPA is required for the unwinding of the replication origin.

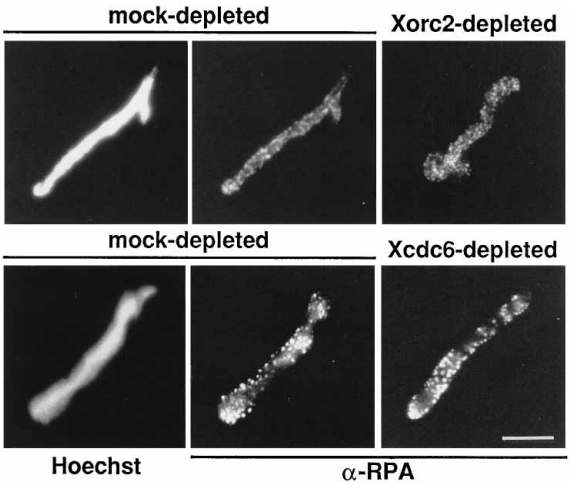

Figure 5. Formation of RPA-Containing Foci Occurs Efficiently in the Absence of Xorc2 or Xcdc6

Demembranated sperm chromatin was incubated for 60 min at 23°C in either mock-depleted (left four panels), Xorc2-depleted (upper right panel), or Xcdc6-depleted (lower right panel) cytosol fractions from interphase egg extracts. The samples were visualized by indirect immunofluorescence with anti-RPA antibodies (right four panels) or by staining with Hoechst 33258 (left two panels). Note that the Hoechst staining is depicted only for the mock-depleted samples. The efficiency of antigen depletion was verified by immunoblotting (Xorc2, 93%; Xcdc6, 94%). Magnification bar, 10 μm.

In order to examine the relationship between Xorc2, Xcdc6, and RPA in the replication process, we performed a series of immunodepletion experiments. In particular, we depleted either Xorc2 or Xcdc6 from the interphase egg extracts, and subsequently examined the association of RPA with chromatin by indirect immunofluorescence with anti-RPA antibodies. For these experiments, we utilized membrane-depleted cytosol from Xenopus egg extracts (Smythe and Newport, 1991). In membrane-depleted cytosol, RPA-containing foci can form normally, but DNA synthesis cannot proceed, since replication requires the presence of a nuclear envelope. Therefore, the RPA foci can be trapped as intermediates with this approach. We treated membrane-depleted cytosol with protein A beads bound to either anti-Xorc2, anti-Xcdc6, or control antibodies. Following removal of the beads by centrifugation, we added sperm chromatin to the various extracts and then assessed the formation of RPA-containing foci by indirect immunofluorescence (Figure 5). Strikingly, both the number and staining intensity of RPA-containing foci on chromatin were unaffected by the depletion of either Xorc2 or Xcdc6. In parallel, we observed that the immunofluorescence staining of chromatin with either anti-Xorc2 or anti-Xcdc6 antibodies revealed a uniform rather than a punctate pattern in control cytosol (data not shown). Together, these experiments indicate that Xorc2 and

Xcdc6 are not concentrated in RPA-containing foci. Furthermore, RPA-containing foci can form efficiently in the absence of either Xorc2 or Xcdc6.

Recombinant Xcdc6 Cannot Rescue the Replication Defect of Intact Nuclei That Had Been Assembled Previously in Xcdc6-Depleted Extracts

The above findings indicate that Xcdc6 plays an essential and early role in DNA replication. Furthermore, the Xcdc6 protein is associated with the nuclear envelope in postreplicative nuclei. In order to examine further the role of the nuclear envelope in Xcdc6 function, we asked whether recombinant His6-Xcdc6 protein could rescue the replication defect in Xcdc6-depleted extracts if nuclear envelope assembly around chromatin were allowed to proceed to completion prior to the addition of the His6-Xcdc6 protein.

For this experiment, we first treated interphase extracts with protein A beads bound to either anti-Xcdc6 or control antibodies (Figure 6). As described previously (see Figure 1), when we added sperm chromatin to these Xcdc6-depleted extracts, it could not undergo DNA replication (5% of control; Figure 6A, compare lanes 1–3 with lanes 7–9). Furthermore, this defect could be reversed if recombinant His6-Xcdc6 protein was added along with sperm chromatin at the beginning of the replication assay (data not shown; see Figure 1). In a variation of this experiment, we asked whether His6-Xcdc6 could rescue the Xcdc6-depleted extracts if it were added 60 min after the sperm chromatin, by which time assembly of the nuclear envelope around the chromatin is complete. The recombinant His6-Xcdc6 protein could not rescue the replication defect of nuclei that had been preassembled in an Xcdc6-depleted extract (Figure 6A, lanes 10–12; Figure 6B, column d). Furthermore, His6-Xcdc6 added at 60 min could not trigger a second round of DNA synthesis in nuclei that had undergone replication in a control extract (Figure 6A, lanes 4–6; Figure 6B, column b). To exclude the possibility that the extracts might have been inactivated during the 60 min incubation at room temperature, we added both His6-Xcdc6 and sperm chromatin to an Xcdc6-depleted extract at 60 min. We observed that this extract could replicate the freshly added sperm chromatin efficiently (Figure 6A, lanes 13–15; Figure 6B, column e). Collectively, these experiments indicate that the formation of the nuclear envelope around sperm chromatin prevents exogenously added Xcdc6 protein from fulfilling its essential function in DNA replication. This observation suggests that Xcdc6 and/or another essential replication factor that collaborates with Xcdc6 cannot cross the nuclear envelope.

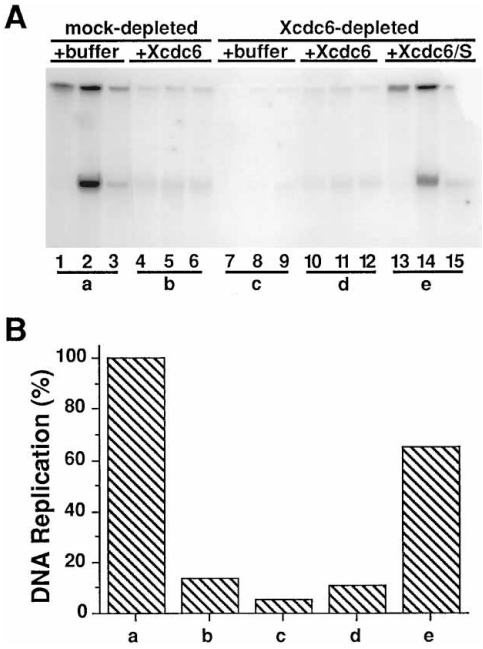

Figure 6. Recombinant His6-Xcdc6 Cannot Rescue the Replication Defect in Preassembled, Xcdc6-Depleted Nuclei

(A) Replication of demembranated sperm chromatin was determined in mock-depleted (lanes 1–6) or Xcdc6-depleted (lanes 7–15) interphase extracts. Demembranated sperm chromatin was added immediately following immunodepletion (lanes 1–12). To these samples, either control buffer was added immediately following depletion procedure (lanes 1–3 and 7–9) or His6-Xcdc6 was added following a 60 min incubation at 23°C (lanes 4–6 and 10–12). Aliquots were pulse labeled with [α-^{32}P]dCTP at various times; each lane represents the DNA synthesis that occurred in successive 30 min intervals after warming to 23°C (0–30 min, lanes 1 and 7; 30–60 min, lanes 2 and 8; 60–90 min, lanes 3, 4, 9, 10, and 13; 90–120 min, lanes 5, 11, and 14; and 120–150 min, lanes 6, 12, and 15). As a control, both His6-Xcdc6 and sperm chromatin (+Xcdc6/S) were added to a Xcdc6-depleted extract at 60 min (lanes 13–15).

(B) Quantitation of the total chromosomal replication for the extracts shown in part (A) over a 90 min period following addition of buffer or His6-Xcdc6. The mock-depleted (columns a and b) or Xcdc6-depleted (columns c–e) extracts were incubated in the presence of control buffer (columns a and c) or purified His6-Xcdc6 (columns b, d, and e). The efficiency of Xcdc6 depletion was verified by immunoblotting (94%).

Discussion

In this study, we have isolated a Xenopus Cdc6 homolog (Xcdc6) and examined its potential role in regulating the replication of vertebrate chromosomes. By immunodepleting this protein from Xenopus egg extracts, we have demonstrated that Xcdc6 is essential for chromosomal replication in this cell-free system. Xcdc6 appears to act at an early step in the replication process, since Xcdc6-depleted extracts can carry out elongation synthesis on single-stranded DNA templates.

We have also examined the localization of the Xcdc6 protein throughout the cell cycle with anti-Xcdc6 antibodies. In addition, we have examined the relationship between Xcdc6 and other critical replication proteins such as Xorc2 and Xmcm3 in order to assess how these factors might collaborate in the replication process. Our studies indicate that Xorc2, Xcdc6, and Xmcm3 are not associated with Xenopus chromatin during M phase. Following inactivation of the Cdc2-cyclin B complex at the end of mitosis, these three proteins bind to chromatin upon entry into interphase. In time-course experiments, these proteins appear to bind to chromosomal DNA with comparable kinetics. However, immunodepletion studies strongly suggest that there is a strict order in which these polypeptides associate with prereplicative chromatin. In particular, we observe that neither Xcdc6

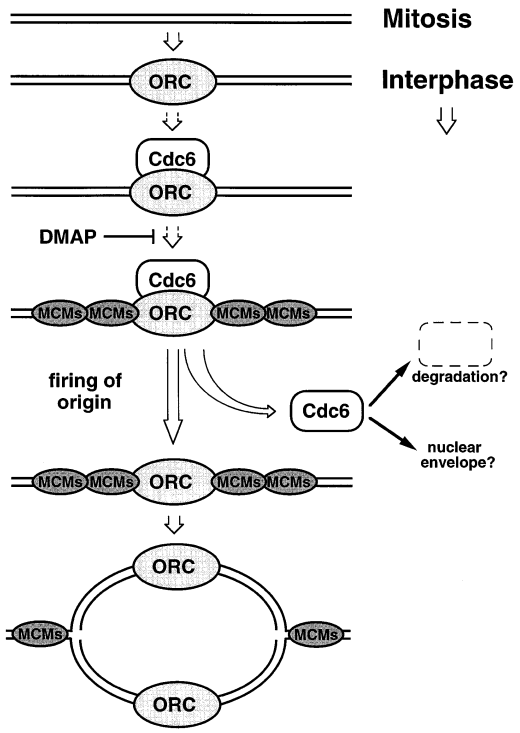

Figure 7. Model Summarizing the Role of Xcdc6 in DNA Replication
See Discussion for details.

nor Xmcm3 can bind efficiently to chromatin lacking the Xorc2 protein and presumably the other components of the Xenopus origin recognition complex. Furthermore, Xorc2 can bind to Xcdc6-depleted chromatin, whereas the Xmcm3 protein requires the Xcdc6 protein for binding. Collectively, these studies appear to indicate that Xorc2, Xcdc6, and Xmcm3 associate with chromatin sequentially prior to a replication cycle.

Once replication begins in assembled nuclei in Xenopus egg extracts, the fates of the Xorc2, Xcdc6, and Xmcm3 differ significantly. As indicated by indirect immunofluorescence, Xorc2 remains associated with chromatin throughout interphase (Figure 7). Conversely, as described by several laboratories, the Xmcm3 protein appears to be dislodged from chromatin as various polymerases copy the DNA template, since the polymerase inhibitor aphidicolin blocks the dissociation of Xmcm3 (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995a). Finally, as described in detail in this study, the behavior of Xcdc6 during a replication cycle is distinct from both Xorc2 and Xmcm3. Our indirect immunofluorescence studies indicate that Xcdc6 is eliminated from chromatin during or shortly after the initiation of DNA synthesis at replication origins. The most persuasive evidence for this view is that Xcdc6 cannot be found associated with chromatin in the presence of aphidicolin. This behavior contrasts dramatically with that of the Xmcm3 protein and suggests that Xcdc6 disappears from the chromatin before replicative polymerases begin to copy the DNA that has been unwound near replication origins (Figure 7).

In nuclei that have begun or completed S-phase, we observe that the nuclear envelope stains strongly with anti-Xcdc6 antibodies by indirect immunofluorescence. Further studies will be required to address the issue of whether individual Xcdc6 molecules shuttle from replication origins to the nuclear envelope following the initiation of DNA synthesis (Figure 7). It should be noted that there is a large excess of the Xcdc6 protein above that required for DNA replication during early embryogenesis. In particular, less than 10% of the Xcdc6 protein is recruited to the chromatin under the conditions of our in vitro replication experiments, in which we have utilized ~1000 nuclei for each egg equivalent of cell-free extract. Thus, although the total abundance of Xcdc6 does not vary discernibly during early embryogenesis, it is conceivable that the small fraction of Xcdc6 associated with chromatin would be subject to degradation following the initiation of replication (Figure 7). If this were the case, the Xcdc6 associated with the nuclear envelope in postreplicative nuclei would presumably arise from the cytosolic pool of Xcdc6 that had not associated initially with the chromatin upon nuclear assembly. In any event, the chromatin in postreplicative nuclei shows a strongly reduced binding of Xcdc6. Since the Xcdc6 protein is absolutely required for DNA replication, this chromatin could not undergo a second round of replication until Xcdc6 would bind to the chromatin at the end of the next mitosis.

Significantly, the Xmcm3 protein also disappears from chromatin during S-phase, but apparently at a later time, since aphidicolin inhibits the dissociation of Xmcm3 but not Xcdc6 from chromatin (Kubota et al., 1995; Madine et al., 1995b; Chong et al., 1996). Upon dissociation, Xmcm3 can be found throughout the soluble compartment of the nucleus, apparently in a state in which it cannot bind to chromatin. This observation can be explained by our finding that chromatin in postreplicative nuclei also lacks the Xcdc6 protein, which is essential for Xmcm3 to bind to the DNA. Thus, at least two essential replication factors, Xcdc6 and Xmcm3, are absent from postreplicative chromatin, thereby preventing a second aberrant round of DNA replication in the same cell cycle.

An important question is how the firing of replication origins is triggered at the beginning of S-phase. Insights from genetic and DNA footprinting studies in yeast have led to the following view of a replication cycle (Diffley and Cocker, 1992; Dahmann et al., 1995; Nishitani and Nurse, 1995; Cocker et al., 1996; Jallepalli and Kelly, 1996). In budding yeast, ORC is bound to replication origins throughout the cell cycle (Diffley and Cocker, 1992). The Cdc6 protein associates with the origin at the end of mitosis, when the cyclin B-associated Cdks are inactivated (Cocker et al., 1996). Later, at the beginning of S-phase, kinase activities associated with Cdc28 and possibly Cdc7 presumably phosphorylate one or more proteins at the origin, which leads to the commencement of replication (Coverley and Laskey, 1994; Heichman and Roberts, 1994). At some point thereafter, the Cdc6 protein disappears from the origin and undergoes degradation. During the S and G2 phases, active cyclin B-associated kinases prevent any Cdc6 protein from binding again to the origin (Dahmann et al., 1995). It will be important to assess this model directly by examining the association of budding yeast Cdc6 and

fission yeast Cdc18 with chromatin and other replication factors during the cell cycle.

In the Xenopus system, the Xorc2 and Xcdc6 proteins show similar, but not identical, properties to those that have been established or predicted for their yeast counterparts. On the one hand, we have provided direct biochemical evidence that Xcdc6 does associate with chromatin at the end of mitosis and furthermore disappears from the DNA upon the initiation of replication. However, one striking difference is that Xorc2 cannot bind to chromatin at M phase in Xenopus extracts. Presumably, the other components of the putative Xenopus ORC are also absent from chromatin at M phase, although it is formally possible that the Xorc2 subunit could dissociate from the other ORC components at this time. The apparent absence of Xenopus ORC from chromatin at M phase suggests that its ability to bind to the origin DNA is regulated by the cell cycle. This difference may be due to the fact that chromosomes of Xenopus and other vertebrates undergo more extensive condensation at mitosis than in yeast. At the end of mitosis, when the chromatin decondenses and the Cdc2-cyclin B complex undergoes inactivation, both Xorc2 and Xcdc6 can reassociate with chromatin.

Following the association of Xorc2 with Xenopus chromatin, the Xcdc6 and Xmcm3 proteins bind shortly thereafter. At some point, S-phase promoting factor (SPF) presumably triggers the firing of the replication origins. In the Xenopus system, the Cdk2–cyclin E complex is necessary and perhaps sufficient to fulfill the role of SPF (Fang and Newport, 1991; Strausfeld et al., 1994; Jackson et al., 1995; Yan and Newport, 1995). The critical targets of Cdk2–cyclin E are not known but Xcdc6 and/or components of the Xenopus ORC are excellent candidates. Phosphorylation of Xcdc6 by Cdk2-cyclin E could lead to both the firing of the origin and the subsequent elimination of Xcdc6 from the origin. Xcdc6 does possess a number of excellent potential Cdk phosphorylation sites, but further studies will be required to address the issue of whether phosphorylation at these sites occurs in vivo and regulates the function of Xcdc6.

One strong advantage of the Xenopus egg system for the study of DNA replication is that the reconstituted nuclei are relatively large (~30 μm in diameter), which makes it feasible to visualize the chromatin and associated proteins by immunocytochemical methods with considerable resolution. Previous studies showed that the single-stranded DNA binding protein RPA associates with several hundred discrete foci throughout reconstituted nuclei in Xenopus egg extracts (Adachi and Laemmli, 1992, 1994; Jackson et al., 1995; Yan and Newport, 1995). These foci form just after mitosis at the beginning of interphase. These structures have also been referred to as prereplication centers, since the incorporation of biotinylated dUTP into DNA at the beginning of replication appears to take place preferentially in these areas (Adachi and Laemmli, 1992; Yan and Newport, 1995). One prevailing model is that strands of DNA are spooled through these static replication centers containing RPA and other replication proteins. It has been estimated that 300–1000 strands of DNA would have to pass through these centers continuously throughout S phase in order to achieve the replication of the entire genome (Mills et al., 1989; Cox and Laskey, 1991; Fang and Newport, 1993).

We believe that our studies of Xcdc6 and Xorc2 in Xenopus egg extracts provide a significant perspective on the topology of the replication process in eukaryotic nuclei. In particular, we find that Xcdc6 and Xorc2 are not concentrated in RPA foci and that RPA foci can form in extracts lacking either the Xcdc6 or Xorc2 protein. These observations indicate that the vast majority of potential replication origins are not concentrated in these focal structures. One explanation could be that only a fraction of these potential origins are utilized during each replication cycle (see DePamphilis, 1993). According to this scenario, this subset of origins would be localized in or recruited to the RPA foci at the beginning of S phase. An alternative view is that most of the initiation events do not occur in the RPA foci. Ultimately, the immunocytochemical visualization of various replication proteins throughout the replication process will contribute greatly to an understanding of the topology of DNA replication in eukaryotic nuclei.

In conclusion, we have identified a Xenopus Cdc6 homolog that collaborates with the Xorc2 and Xmcm3 proteins in chromosomal replication. Based on the experiments described here, Xcdc6 appears to play a crucial role in regulating the timely and faithful initiation of DNA replication in a cell-free system that recapitulates the duplication of vertebrate chromosomes. Further studies of the regulation of Xcdc6, its intracellular localization throughout the cell cycle, and its association with other replication factors will be valuable for understanding the mechanisms of how animal cells replicate their chromosomes properly.

Experimental Procedures

Isolation of a cDNA Encoding a Xenopus Cdc6-like Protein

An internal Xenopus Cdc6 fragment was cloned by PCR amplification using degenerate primers specific to regions of high amino acid sequence similarity shared between the S. cerevisiae Cdc6 and S. pombe Cdc18 proteins (residues 196–203 and 455–462). The 5′ and 3′ primers were CGCGGATCCGG(I/C)(C/G)C(I/C)CC(I/C)GG(I/C)AC(I/C)GG(I/C)AA(A/G)AC and CGGGGTACCGTICA(C/I)AI(C/I)AT(C/I)(G/A)(C/I)(T/C)TT(T/C)TG(T/C)TG, respectively. The 5′ ends of each primer contain nine extra nucleotides (underlined) that provide restriction sites for BamHI or KpnI. PCR reactions (50 μl) contained Xenopus oocyte cDNA (15 ng; generous gift of Dr. P. Mueller), primers (50 pmol each), and the buffer conditions recommended by the manufacturer. The reactions were initiated by heating to 85°C before the addition of *Taq* DNA polymerase (Perkin-Elmer) and cycled using the profile described in Mueller et al. (1995) except the annealing temperature was 52°C. After electrophoresing an aliquot of the reaction mixture in a 1.5% low melting point agarose gel, we identified an ~800 base pair fragment that was subcloned into the TAII cloning vector (Invitrogen). This ~800 base pair fragment was radiolabeled and used to screen a Xenopus oocyte cDNA library (Mueller et al., 1995) by colony hybridization. A full-length clone was selected for further study and subcloned into a modified Bluescript SK⁻ vector (Stratagene) containing three additional restriction sites (NdeI, NheI, and AatII) that had been inserted in the polylinker at position 655. Nested sets of unidirectional deletions were constructed in both directions, using the Erase-a-Base system (Promega Biotech), and sequenced on both strands by standard methods.

Plasmid Construction for Production of Histidine-Tagged Xcdc6 Protein

To synthesize full-length recombinant Xcdc6 protein, the initiation codon was converted to an NdeI restriction site by PCR. The 5′

primer, GGTCCATATGCCAAGCACCAGGTCTCGGTCTCAAAG, is complementary to the beginning of the Xcdc6 open reading frame and contains an additional 10 base pairs which encode an NdeI restriction site (underlined). The 3′ primer was GCTGATCCATCTCAT CCAACACC. PCR reactions (50 μl) contained template (50 ng), primers (0.5 pmol each), *Pfu* DNA polymerase (Stratagene), and the buffer supplied by the manufacturer. The reactions were cycled 30 times as follows: 94°C, 1 min; 60°C, 3 min; and 72°C, 5 min. The product of the PCR reaction was used directly for subcloning into TAII (Invitrogen). The sequence of the modified Xcdc6 was confirmed. The full-length Xcdc6 coding region was subcloned into pVL1393N-HIS (Tang et al., 1993) in two fragments: a 92 bp NdeI-BglII fragment (5′ end) and a 2.1 kb BglII-EcoRI fragment (3′ end). The resulting vector, pVL-HIS-Xcdc6, encodes full-length Xcdc6 protein (His6-Xcdc6) with a six-histidine tag at its N-terminal end.

Production and Purification of Xcdc6 Protein

A recombinant baculovirus His6-Xcdc6 was isolated by standard procedures (Invitrogen). Sf9 insect cell lysates containing His6-Xcdc6 were prepared (Desai et al., 1992) and the histidine-tagged protein was immediately bound to nickel-iminodiacetic acid Sepharose (Pharmacia) and purified as described (Kumagai and Dunphy, 1995). Proteins that were added to Xenopus extracts were dialyzed against 10 mM HEPES–KOH (pH 7.4), 150 mM NaCl, 0.1 mM dithiothreitol.

Antibody Production

Rabbits were immunized with purified His6-Xcdc6 protein, and antibodies were purified by affinity chromatography (Harlow and Lane, 1988) on CNBr-activated Sepharose-4B (Pharmacia) containing covalently bound His6-Xcdc6. Antibodies against Xorc2 were prepared as described (Carpenter et al., 1996). The anti-Xmcm3 (Madine et al., 1995a), anti-RPA (Fang and Newport, 1993), and anti-lamin L_{III} (Benavente et al., 1985) antibodies were generous gifts of Drs. R. Laskey (Wellcome/CRC Institute), J. Newport (U. C. San Diego), and G. Krohne (German Cancer Research Center, Heidelberg, Germany), respectively.

Immunofluorescence Microscopy, Immunodepletion, and Chromatin Immunoblots

Xenopus cytostatic factor (CSF)–arrested egg extracts (blocked in M phase) were prepared from unactivated eggs as described (Murray, 1991). Xenopus CSF extracts were supplemented with demembranated sperm chromatin (1000 nuclei/μl) and $CaCl_2$ (0.4 mM). In some cases, extracts were arrested in interphase with either cycloheximide (100 μg/ml), aphidicolin (100 μg/ml), or DMAP (3 mM). In one experiment, a cycloheximide–treated extract was driven into mitosis by supplementing the extract with purified Δcyclin B (Kumagai and Dunphy, 1995; kindly provided by D. Patra). At various times following activation, extracts were diluted, fixed, and layered on a sucrose cushion, and the nuclei pelleted onto coverslips (Mills et al., 1989; Yan and Newport, 1995). Indirect immunofluorescence was performed using either goat anti-mouse IgG conjugated with tetramethylrhodamine isothiocyanate (Cappel) or goat anti-rabbit IgG conjugated with either fluorescein isothiocyanate (Cappel) or Texas red (Jackson ImmunoReseach). In some cases, the chromatin was stained with Hoescht 33258 to visualize the DNA. Samples were viewed on a Zeiss Axiophot equipped with a 63× objective and photographed at identical magnification and exposure times. For visualization of replication foci, demembranated sperm chromatin (1000 nuclei/μl) was incubated in membrane-depleted S-phase extracts (Smythe and Newport, 1991) and processed for indirect immunofluorescence as above. Immunodepletions were performed on either interphase extracts (15 min postactivation) or membrane-depleted S-phase extracts using protein A–agarose containing either anti-Xcdc6, anti-Xorc2, or control rabbit anti-mouse IgG (Cappel) antibodies (10 μg antibody/100 μl extract). All experiments were performed using double-depleted extracts. Typically, two such sequential immunodepletions removed 90%–98% of either Xcdc6 or Xorc2. In order to identify chromatin-associated proteins by immunoblotting, Xenopus egg extracts containing sperm chromatin were diluted in a detergent containing buffer and centrifuged on a sucrose cushion as described (Kubota et al., 1995). The pelleted chromatin and the detergent-insoluble matrix that surrounds the chromatin were resuspended in gel sample buffer and processed for immunoblotting.

Miscellaneous Methods

Replication of sperm chromatin (1000 nuclei/μl) or M13 DNA (6 ng/μl) was monitored by agarose gel electrophoresis following pulse labeling with [α-^{32}P]dCTP (Dasso and Newport, 1990). Protein concentrations were determined using the BioRad protein assay kit. Immunoblotting was performed as described (Coleman et al., 1993). Quantitation of immunoblots and replication assays was performed using a PhosphorImager (Molecular Dynamics).

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